Research Article

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IN-VITRO SCREENING OF TOXIC BIOACTIVE FRACTIONS OF CYANOBACTERIAL SP. AND CHARACTERIZATION OF ITS METABOLITES THROUGH HIGH PRESSURE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

Cyanobacteria is a normal resident of any freshwater bodies, however the recent threats caused by the blooms to the environment, aquatic animals has increased the focus in screening of toxic form of cyanobacteria. Hence the present study is focused on the identification of cytotoxicity of the isolated species of cyanobacteria, *Oscillatoria amphibia*. The invitro cytotoxicity on Ehrlichs ascites carcinoma cells revealed that ethanolic extract or aqueous extract of *O.amphibia* showed cytotoxicity even at 5µg concentration. It was observed through HPTLC chromatogram, that extracts contain molecules having absorbance maxima of mycrocystin molecules ranging with a λ max of 200-223nm. The overall significance of the study recommends the invitro cytotoxicity study as the rapid screening method for the identification of cytotoxic forms of cyanobacteria. Hence the quarantine measures for the eradication of the toxic blooms could be warranted.

Keywords: *Oscillatoria amphibia*, HPLC, HPTLC, Ehrlichs ascites carcinoma, Microcystin.

INTRODUCTION

Cyanobacteria normally termed as Blue green algae occurs commonly in the eutrophicated regions of rivers, estuaries etc [1]. They are distinctly known either for their therapeutically active compounds or for their potent toxicity. Basically the Bloom forming microorganism(s) posses the typical characteristics of toxin release and thereby create a noxious environment to the waterbodies. The cyanotoxins are divided into three classes based on the chemical structure; they are cyclic peptides, alkaloids, and lipopolysaccharides. Microcystins and nodularins are cyclic peptides containing seven and five amino acids respectively. There are more than 65 structural variants of microcystins differing in modifications to the peptide backbone or the type of amino acids incorporated into the microcystin [2]. The general structure of microcystins can be summarized as cyclo-D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-Glu^6-Mdha^{7}where X and Z are variable L-amino acids (Fig1). Microcystin R.R, microcystin.L.R, microcystin W.R are the mostly available form of toxins found in the fresh water blooms. Various analytical techniques have been developed such as HPLC, ELISA, mass spectrum [3], LCMs for the detection of toxins from cyanobacteria and the lethal rate of the toxicity was checked through the *in-vivo* studies such as mouse bioassay technique [4] or through invitro cell culture techniques. In the present study author has screened for the presence of toxin in the extracts of *O. amphibia* by invitro cytotoxicity assay in EAC cells. HPTLC of extracts were performed to ascertain the toxin present in the extract,

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which is again a powerful separation technique to separate the specific toxins based on their R_f and identify based on their typical absorption maxima.

Figure 1: The structure of Mycrocystin, with add a region.

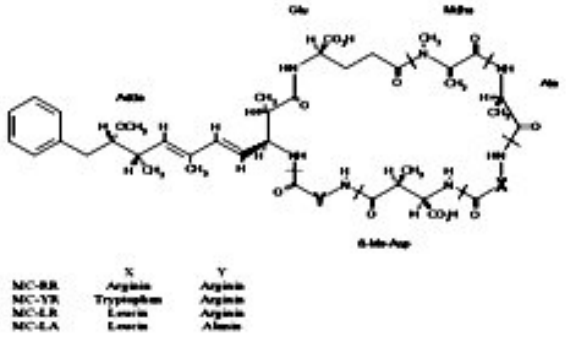

Fig : 1 Structure of Microcystin withdifferent regions representing X andY. The variantsofmicrocystinexists asa resultofchange in theamino acidcontent in the position

Figure 2: Microscopic examination of pure form of Oscillatoria *amphibia* isolated from the fresh water forms. Observed under 40 X magnification in compound microscope.

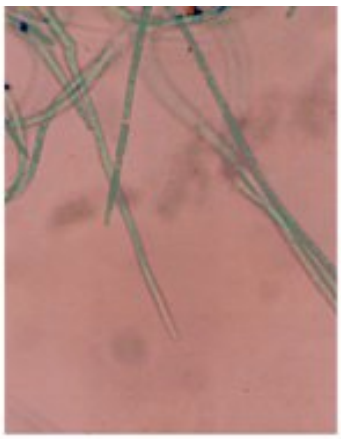

Fig : 2 *Oscillatoria amphibia* isolated from the fresh water sources

EXPERIMENTAL

1. Extraction and isolation of crude compounds

The cyanobacteria was isolated and cultivated in BG-11 medium and the strain isolation was done by streaking on the BG-11 agar plates the colonies were picked up and again scaling up was done in BG-11 medium [5]. The cyanobacterium was harvested through membrane filtration and the cells were collected washed thoroughly in tap water [6]. The mats were then subjected to air drying. The dried material was subjected to extensive maceration in ice cold mortar and pestle using ethanol or water at a proportion of 1:5 W/V. The extract obtained was filtered and concentrated by vacuum evaporation under reduced pressure.

2. In vitro cytotoxic assay

The extracts were screened for the bioactivity in Ehrlichs ascites carcinoma cells. The cytotoxicity was determined through trypan blue dye exclusion method. The EAC cells were aspirated from the peritoneal cavity of the Swiss albino mice injected with the EAC cells [7]. The ascites were suspended in PBS buffer pH 7.2 and the cell number of $10x10^6$ cells/ml was maintained, ascertained for their viability of atleast 90%. Different concentrations of extracts was incubated in the EAC cells $1x10^6$ cells/ml for 3 hours and 1ml of trypan blue was added over it and cytotoxicity of the cells were enumerated under the assay mixture 1ml using phosphate buffer saline and incubated at 37°C for 3hrs and the number of nonviable cells were determined by using haemocytometer [8,9].

3. HPTLC Analysis of microcystin fractionate

High performance thin layer chromatography is the most simplest and sensitive technique for the detection of compounds based on their absorption maxima. The activated precoated silica gel plates were spotted with 2µl of the extract and the plates were kept in the solvent (T. butanol: acetic acid : water 10:1:1) saturated chambers [10]. The chromatographed plates were further developed with iodine vapours and the spots were detected using the camag software provided with unit.

RESULTS AND DISCUSSION:

Cyanobacteria are pathogenic prokaryotes known for producing a high variety of cyclic hepatotoxic peptides in fresh and blackish water. Prominent members of these toxins are microcystin LR (MC LR) and nodularin (Nod), which are under suspicion to cause cancer [11, 12]. In the present study authors had screened for the presence of toxins in Ehrlichs ascites carcinoma cells. The viability of these cells in the presence of these cyanobacterial extracts was enumerated using

the dye exclusion assay. It was observed from the study that cell death of EAC in aqueous extract of *O.amphibia* was found to be 100% at a concentration of 100µg, similarly the cell death was also 100% in the case of ethanolic extract (Fig 4). However the viability of untreated cells was found to be intact for 2 hours. The percentage of cytotoxicity decreased with the descending concentrations of the extract, however the minimum cytotoxic dosage was observed even at 5µg concentration(s) of aqueous or ethanolic extract (Fig 3.a & 3.b). The most characteristic feature of cytotoxicity such as membrane blebbing was clearly observed in the presence of ethanolic extracts of *O. amphibia*. Several methods were employed to assess the toxicity of cyanobacterial toxins based on their toxicity levels such as hepato toxicity, neurotoxicity, cytotoxicity or enzyme inhibiting activity, mouse bioassay is being employed alone for the overall detection of toxicity in the water blooms. Hence in the present study, Ehrlichs ascites carcinoma cells were used for the screening of toxicity. Similar studies for the screening of invitro cytotoxicity were performed on HepG2 cell lines [13, 14]. Various analytical methods have been reported for the detection of these cyclopeptides, and these are mainly based on liquid chromatography combined with mass spectrometric techniques [15, 16]. The present study is focused on the screening of toxicity of *O amphibia* (Fig 2) isolated from the fresh waters of Tamilnadu, The cauvery river. The authors adopted the HPTLC technique for this study. It was observed from the study that, ethanolic extract of *O. amphibia* showed a HPTLC fingerprint of molecules exhibiting an absorption maxima of λ= 222nm, the microcystins have an absorption maxima of λ= 222nm (Fig 5 a., Table 1). Similar studies carried out by Codd et al revealed that cyclopeptides when separated on the HPTLC plates associated with IR- Maldi-o-TOF Ms exhibited the λ= 223 nm. Similar studies revealed the use of RP – HPLC for the detection of microcystin through PDA detector based on their typical UV absorption maxima. Studies also revealed the importance of HPTLC and TLC methods for the detection and purification of microcystins[17]. Ojanpera et al., 1991 claimed TLC method of detection of microcystin to be the rapid method [18]. It was also observed from the HPTLC chromatogram, there are some molecules, with λ=200nm, However the nature of this molecule was found to be obscure (Fig 5b. Table 2). The Aqueous extract of *O.amphibia* was found to possess the molecules that have an absorbance maxima of λ=400nm.The overall chromatography mapping revealed that some of the molecules are identical in both the extracts. These molecules had similar absorption maxima and R_f values respectively.

Table 1: The fractions No. 1-3 have the absorption maxima in UV range and the fractions 4, 5 and 6 have absorption maxima in visible region.

S. No	R_f values	Absorption maxima
1	0.04	222
2	0.05	222
3	0.17	200
4	0.79	400
5	0.83	400
6	0.89	400

Table 2: The fractions No. 1-7 fhave the absorption maxima in UV range and the fractions 8 and 9 have visible absorption maxima in visible region. The area under the HPTLC chromatogram was illustrated blue using Adobe photoshop.

S. No	R_f values	Absorption maxima
1	0.06	200
2	0.13	200
3	0.18	200
4	0.23	200
5	0.37	200
6	0.48	260
7	0.62	260
8	0.75	400
9	0.85	395

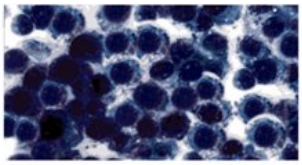

Figure : 3 a. EAC cells treated with the extractsof*O. amphibia* and their dye uptake 5 (b).

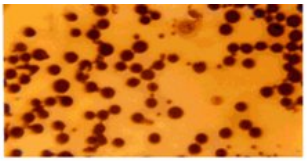

Figure : 3 b. Ehrlichs Ascites carcinoma cells- Normal cells

Figure 3.a & 3 b: Ehrlich's Ascites Carcinoma (Viable Cells) and cells treated with the extract of the *Oscillatoria amphibia*. The cells were collected from the peritoneal cavity and the viability was checked initially. The cells absorbing the dye (Trypanblue) due to the invitro cytotoxicity. Variation in the shape of cell after the treatment with the extract of *Oscillatoria amphibia* (membrane blebbing is the common phenomenon observed).

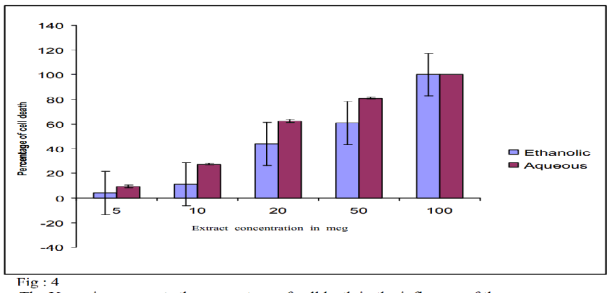

Fig : 4
The Y –a xis represents the percentage of celldeath in the influence of the toxic molecules in the extract . X- axis represents the concentration of the extract ,unit expressed in µg
The values are expressed as Mean ± S.E

Figure 4: *In vitro* Cytotoxic Activity of Ethanolic and Aqueous Extract of Oscillatoria *amphibia.* Percentage of cell death is calculated and plotted against the different concentration of the ethanolic as well as aqueous extract. Data expressed as Mean ± S.E

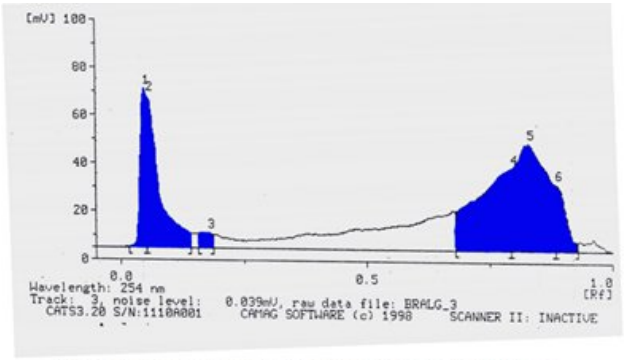

Fig 5 a: Aqueous extract of *O. amphibia* fractionated on the HPTLC plates

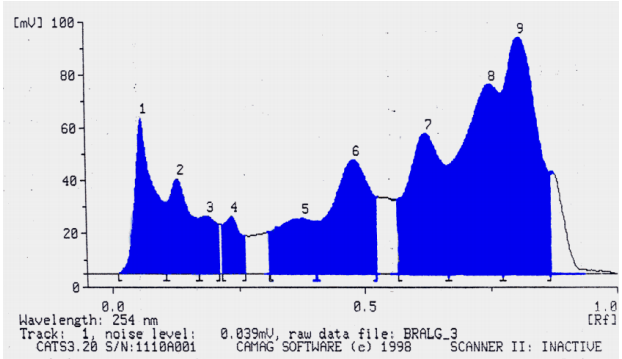

Fig 5 b: Ethanolic extract of *O. amphibia* fractionated on the HPTLC plates

Figure 5a & 5 b: HPTLC Chromatogram of Ethanolic Extract and Aqueous extract of *Oscillatoria amphibian*

CONCLUSION

The study concludes that, from the isolated strain of Cyanobacteria was found to contain the cytotoxic property like microcystins. It was observed from the previous studies that the limit of detection of these cyclopeptides was found to be 5ng for U.V spectroscopy and 3ng for the mass spectrometric analysis. The overall significance of the study implies the sensitivity of the *in-vitro* cytotoxicity test and HPTLC technique which is in par with other sophisticated analytical techniques employed in the identification of potent toxins.

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